

Applicants: Fahey, et al.
Application No.: 09/530,370
Filed: July 6, 2000
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In the Specification

Please amend the paragraph beginning at line 6 on page 43 to read as follows:

B 1
A membrane-based assay based on the immunoassay architecture described in Example 4 was developed for detecting MSH production by bacterial colonies grown on solid media. The following protocol is typical for a single 100 mm Petri dish. All incubations were done at room temperature on an orbital platform shaker set to 90 rpm unless otherwise noted. Different bacteria, including two strains of *Mycobacterium smegmatis* (mc²-6 and mc²-155) and several non-MSH-producing species (*Escherichia coli* HB101, *Enterococcus faecalis* ATCC 29212, and *Streptococcus mutans* ATCC 33402) were grown as separate streaks on a single agar dish. A supported nitrocellulose membrane circle (NITROPURE®, 0.45 µm porosity, 81 mm diameter) was marked with a pencil for orientation on the plate, and pre-soaked in TBS. Excess TBS was drained from the membrane, a freshly-made solution of Pierce IMJECT® maleimide-activated BSA (265 µg in 13.3 ml TBS, to give 5 µg/cm² loading) added, and the membrane incubated for 30 min. Excess liquid was drained from the membrane, which was then laid onto the surface of the bacterial plate with care to avoid bubbles or smearing of the bacterial colonies. The membrane was lifted carefully and laid bacteria-side up for 1 h in a clean glass Petri dish containing a solution of *N*-acetylglucosaminidase (3.1 units in 10 ml TBS adjusted to pH 4.2 with acetic acid). The membrane was next washed briefly with TBS to remove adhering cells and washed with 10 ml TBST. Excess liquid was drained and the membrane incubated in 10 ml 2% fish skin gelatin in TBS for 2 h. The membrane was drained, 10 ml affinity-purified anti-MSH IgG solution (containing ~18 µg total protein) added and incubated overnight at 4 °C on an orbital platform shaker set to 60 rpm. The antibody solution was aspirated and the membrane

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washed 3 times in TBST (10 ml and 10 min for each wash). Excess liquid was drained, 10 ml of secondary antibody (goat anti-rabbit [whole IgG] F(ab')₂ fragments conjugated to bovine intestinal alkaline phosphatase, diluted 1:15000 in TBS) added, and the membrane incubated for 1 h. The membrane was drained, washed twice in TBST and thrice in TBS (10 ml and 5 min each wash). Development was with BCIP-NBT (SigmaFAST®). After thoroughly washing in distilled water, the blot was air-dried. MSH-containing bacteria are revealed as dark purple stains; only the two strains of *M. smegmatis* produced positive signals (Figure 3).

Please amend the two paragraphs that begin at line 15 of page 44 to read as follows:

B. Since reaction of typical cellular constituents with maleimide-BSA is largely limited to thiols, it was considered important to ascertain whether any typical biological thiols would produce false-positive assay results. The following thiols were tested in the ELISA protocol described above in Example 4: L-cysteine, glutathione, pantetheine, and coenzyme A. At the 10 pmol level all of these gave negative results indicating that these are not recognized by the anti-mycothioli antibody (Figure 4).

In order to test whether the anti-mycothioli antibody recognizes component parts of the mycothioli molecule we also tested the ELISA on *N*-acetyl-L-cysteine, L-cysteinyl-glucosamine, (2-L-cysteinyl)amido-2-deoxy- α -D-glucopyranose, and *N*-acetyl-L-cysteinyl-glucosamine (2-*N*-acetyl-L-cysteinyl)amido-2-deoxy- α -D-glucopyranose). The first two gave negative results at the 10 pmol level but the latter compound, which has the structure of MSH with the inositol removed, gave a positive assay with a sensitivity of about 4% of that for MSH (Figure 4).